Agents Actions 38, *Special Conference Issue* (1993)

0065-4299/93/020C80-03 $1.50+0.20/0

Effects of leflunomide (HWA 486) on expression of lymphocyte activation markers

Th. Zielinski[1], H.-J. Müller[2] and R. R. Bartlett[2]

[1] Department of Immunology, University of Mainz, W-6500 Mainz, FRG, [2] Biomedical Research, Immunopharmacology, Hoechst AG, Werk Kalle-Albert, W-6200 Wiesbaden, FRG

Abstract

Leflunomide (HWA 486), an isoxazol derivative, has been shown to be very effective in combating autoimmune diseases and transplantation rejection in a great number of animal models. The main metabolite of leflunomide, A77 1726, is a potent antiproliferative compound. To further elucidate this effect, lymphocytes of healthy human donors were cultured for 24, 48 or 72 h in the presence of PHA or immobilized anti-CD3 antibody. A77 1726 was added at concentrations between 10 and 100 μM. Flow cytometric evaluation of early activation or proliferation markers (IL-2 and transferrin receptors, respectively) showed that their expression was inhibited in a dose-dependent manner by A77 1726. Together with previous data, these experiments indicate that leflunomide not only inhibits the expansion of already proliferating lymphocytes, but also impairs PHA and anti-CD3 antibody triggered activation of quiescent cells. Thus, this compound may exert its effects through influencing two important aspects of an immune response, that is, activation and proliferation of lymphocytes.

Introduction

Leflunomide (HWA 486), an isoxazol derivative, is effective in combating autoimmune diseases and transplantation rejection in a number of animal models [1, 2]. Leflunomide's main metabolite, A77 1726, exhibits strong antiproliferative activity *in vitro*.

To elucidate this effect further, quiescent and non-proliferating human lymphocytes were activated with PHA or immobilized anti-CD3 antibody. This activation leads to interleukin 2 (IL-2) and transferrin receptor expression, where IL-2 receptors can be considered as early activation markers (early G_1 phase) [3]. Moreover, IL-2 and transferrin receptors are essential for clonal expansion of lymphocytes, leading to a potent immune response [3, 4].

In this present study, we investigated the ability of leflunomide to influence the expression of IL-2 and transferrin receptors with the intention of determining a differentiated mode of action concerning its influence on quiescent and proliferating cells.

Address for correspondence: [2] R. R. Bartlett, Immunopharmacology, Hoechst AG, Werk Kalle-Albert, P.O.-Box 3540, W-6200 Wiesbaden, FRG.

Materials and methods

Lymphocyte activation and receptor determination

Blood from healthy, adult donors was drawn by venipuncture. Lymphocytes were isolated by density gradient centrifugation and seeded in 12-well plates ($1-2 \times 10^6$ cells/well). Cells were cultured in cell growth (CG) medium (Camon, Germany) supplemented with 1% human serum and appropriate

Table 1
Effect of A77 1726 on lymphocyte, interleukin-2 receptor expression

	No PHA, anti-CD3 or A77 1726	A77 1726 0.0 *M*	A77 1726 10^{-5} *M*	A77 1726 2.5×10^{-5} *M*	A77 1726 5×10^{-5} *M*	A77 1726 7.5×10^{-5} *M*	A77 1726 10^{-4} *M*
24 h PHA[a]	4.9 ± 0.7	40 ± 7.2	43.4 ± 7.9	33.3 ± 4.5	26.5 ± 10.7*	18.6 ± 8.3**	12.3 ± 7.1***
48 h PHA[b]	4.6 ± 2.2	57.2 ± 12.8	58.3 ± 11.6	49.3 ± 16.5	35 ± 16.2*	18.6 ± 12.4**	13.7 ± 7.8***
24 h anti-CD3[a]	23.9 ± 0.8%	91.4 ± 1.2%	87.4 ± 1.6%	79.9 ± 0.55%*	75.7 ± 1.2%*	58.3 ± 0.9%**	38.5 ± 0.8%***
48 h anti-CD3	25.5 ± 0.7%	388.9 ± 1.1%	336.8 ± 1.3	264.2 ± 1.4%*	245.8 ± 0.7%**	196.3 ± 2.6%**	139.5 ± 3.7%**

[a] Mean ± SD of 3–6 healthy donors, % positive lymphocytes.
[b] Mean fluorescence intensity ± SD%, data from a representative experiment.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 2
Effect of A77 1726 on lymphocyte, transferrin receptor expression

	No PHA, anti-CD3 or A77 1726	A77 1726 0.0 *M*	A77 1726 10^{-5} *M*	A77 1726 2.5×10^{-5} *M*	A77 1726 5×10^{-5} *M*	A77 1726 7.5×10^{-5} *M*	A77 1726 10^{-4} *M*
24 h PHA*	2.8 ± 2.5	26.3 ± 15.5	30.6 ± 17	18.3 ± 10.6	18.9 ± 11.8	16.8 ± 9.1	12.9 ± 7*
48 h PHA**	6.6 ± 5.9	52.1 ± 20.2	46 ± 20.9	27.4 ± 16.1	29.4 ± 16.8	16.6 ± 10.7*	14.7 ± 6*
72 h anti-CD3**	55.5 ± 1%	209.1 ± 1.3%	179.4 ± 1.4%	147.2 ± 0.2%**	134.6 ± 0.4%***	114.4 ± 5.3%*	79.9 ± 4.5%**

* Mean ± SD of 3–6 healthy donors, % positive lymphocytes.
** Mean fluorescence intensity ± SD%, data from a representative experiment.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

dilutions of A77 1726. Anti-CD3 antibodies, of predetermined amounts, were bound to culture plates by overnight incubation in phosphate buffered saline (PBS). Staining of surface antigens was performed according to standard laboratory methods (30 min., 0 °C, 0.1% Na-azide), using Becton/Dickinson antibodies to IL-2 receptor (IL-2-R) α-chain or transferrin receptor. Analysis of receptor expression was conducted with a FACScan cytometer and, on the basis of 10^4 recorded events, analysed by use of FACScan software. Only living cells, determined by propidium dye exclusion (5 μg/ml) and forward/side scatter appearance, were allowed for analysis. The two-tailed Student's *t*-test was used for statistical evaluation of the results.

Results

Expression of IL-2 and transferrin receptors

The expression of both, IL-2 and transferrin receptors, on PHA activated lymphocytes, was inhibited by A77 1726. As shown in Table 1, A77 1726 exhibited a dose-dependent inhibition of IL-2-R expression. Significant inhibition of PHA-induced expression was reached, in 24 h and 48 h cultures with a minimum of 50 μ*M* A77 1726. The inhibitory effect of A77 1726 on the expression of transferrin receptors is shown in Table 2. The data represent the mean percentage of receptor positive lymphocytes of 3 to 6 different healthy donors.

Data showing the effect of A77 1726 on the anti-CD3 antibody triggered activation are presented in Table 1 (IL-2 receptor expression) and Table 2 (transferrin receptor expression). Thus, in every set of experiments, in which lymphocytes were activated, the compound clearly inhibited the expression of activation markers. Toxicity of A77 1726 in these experiments varied greatly between the individual donors, and was increased two-to-three fold at the 100 μ*M* concentration (16.1% ± 8% baseline, 37.2% ± 21% after treatment).

Discussion

Leflunomide is effective in combating autoimmune diseases and transplantation rejection in a number of animal models [1, 2]. The main metabolite of leflunomide, A77 1726, exhibits strong antiproliferative activity *in vitro* [3]. To characterize this effect further, peripheral lymphocytes were activated, with mitogen or anti-CD3 antibody, in the absence or presence of A77 1726. This activation normally leads to the de novo or enhanced expression of a great number of surface antigens. The expression of IL-2 receptors has been shown to be an early activation (early G_1-phase) marker [4]. Using anti-CD3 or PHA to trigger lymphocytes, A77 1726 clearly inhibited the expression of IL-2 receptors in a dose-dependent manner. Preliminary data show that this was also true when leflunomide was given into the mixed lymphocyte culture, but only marginal influence was noted when phorbol myristate was used as the stimulus (data not shown), indicating a possible influence of A77 1726 on signal transducting steps proximal to protein kinase C. Additionally, we observed that A77 1726 inhibited the transition of activated lymphocytes into the blast state. Interestingly, it seems that the compound exerts only marginal influence on the production of lymphokines [1]. From this data we conclude that leflunomide influences signals that are responsible for lymphocytes to enter the cell cycle following appropriate activation.

Together with previous data, dealing with tyrosine kinase inhibition [1] and cell cycle analysis (manuscript in preparation), these experiments indicate that A77 1726 not only inhibits the expansion of proliferating cells, through blocking cell cycle transition in the early S-phase, but also impairs PHA, anti-CD3 and antigen triggered activation of quiescent lymphocytes. Thus, leflunomide may exert its *in vivo* effects through influencing two important aspects of an immune response, lymphocyte activation and proliferation.

References

[1] R. R. Bartlett, M. Dimitrijevic, T. Mattar, T. Zielinski, T. Germann, E. Rüde, G. H. Thoenes, C. C. A. Küchle, H.-U. Schorlemmer, E. Bremer, A. Finnegan and R. Scleyerbach, *Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection.* Agents and Actions *32,* 10–21 (1991).

[2] T. T. Glant, K. Mikecz, R. R. Bartlett, F. Deak, E. Thonar, J. Williams, T. Mattar, K. Kuettner and R. Schleyerbach, *Immunomodulation of proteoglycan-induced progressive polyarthritis by leflunomide.* Immunopharmacol. *23,* 105–116 (1992).

[3] T. A. Waldmann, *The structure, function and expression of interleukin-2 receptors on normal and malignant T cells.* Science *232,* 727–732 (1986).

[4] L. M. Neckers, *Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2.* Proc. Natl. Acad. Sci. USA *80,* 3494–3498 (1983).